

A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium)

Sofie E. De Meyer^{a, b, *}, Kristof De Beuf^c, Bram Vekeman^a, Anne Willems^a

^a Laboratory of Microbiology, Dept. Biochemistry and Microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

^b Centre for Rhizobium Studies, Murdoch University, Western Australia 6150, Australia

^c Fostering Innovative Research based on Evidence (FIRE), Doctoral Schools, Ghent University, Sint-Pietersnieuwstraat 25, B-9000 Ghent, Belgium

ARTICLE INFO

Article history:

Received 22 September 2014

Received in revised form

11 December 2014

Accepted 1 January 2015

Available online 16 January 2015

Keywords:

Endophyte

Legume symbiosis

Root nodules

Rhizobia

ABSTRACT

We analysed the genetic properties of non-rhizobial root nodule endophytes (NRE) isolated from indigenous legumes in Flanders. In total, 654 isolates were obtained from 30 different plant species within the Faboideae legume subfamily. Partial sequencing of the 16S rRNA gene revealed a large diversity of different taxa from the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmibacteria, Flavobacteria and Sphingobacteria. Many of the isolates belonged to the genera *Bacillus* (17.9%) and *Pseudomonas* (15.9%). No symbiosis (*nodC*) or nitrogen fixation related genes (*nifH*) could be detected amongst the isolates, which indicate the endophytic nature of the bacteria. Statistical analysis grouped the investigated plant species into six clusters according to the presence of particular NRE. However, no correlations could be found within these six clusters towards plant tribes or ecoregions the plants had been sampled from. Cluster analysis of the ecoregions according to the presence of NRE, revealed correlations between bacterial genera and those areas. However, groups present in the ecoregions did not correlate with the groups present in the different plant clusters. When combining our previous study on rhizobial diversity recovered from the same sampling campaign (De Meyer et al., 2011) with the current study, 84.1% of the isolates belonged to the traditional rhizobia groups and only 15.9% were NRE. The Loamy ecoregion yielded the lowest number of culturable NRE (8.04%) and the Campine ecoregion the highest number (24.19%). The present study highlights the frequent presence of these NRE in root nodules. The occurrence of certain rhizobia was correlated with the presence of particular NRE, suggesting their presence may not be accidental, however their functions remain unclear at this point.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Rhizobia are soil bacteria capable of symbiosis with legume plants where they can reside in root or stem nodules and perform nitrogen fixation for the host. While traditionally, rhizobia belonged to the genera, *Azorhizobium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* (Sawada et al., 2003), in recent years nitrogen fixing root nodule bacteria have also been described in other Alphaproteobacterial genera, including *Ochrobactrum* (Trujillo et al., 2005), *Methylobacterium* (Sy et al., 2001), *Microvirga* (Ardley et al., 2012; Radl et al., 2014), *Devosia* (Rivas et al., 2003) and

Phyllobacterium (Zakhia et al., 2006). Furthermore, so-called Beta-rhizobia have in the last ten years been described in the Betaproteobacterial genera *Burkholderia* and *Cupriavidus* (Chen et al., 2001; Moulin et al., 2001; De Meyer et al., 2013a; De Meyer et al., 2013b; De Meyer et al., 2014). In addition to strains that can elicit nodules and belong to documented rhizobial species, several other bacterial species have been reported from legume nodules without a clear indication of their role within the host. In the absence of positive nodulation tests, they can be regarded as non-rhizobial endophytes (NRE). These include i.a., Alphaproteobacteria (*Aminobacter* (Estrella et al., 2009), *Ochrobactrum* (Zurdo-Pineiro et al., 2007; Imran et al., 2010), *Methylobacterium* (Palaniappan et al., 2010), *Devosia* (Bautista et al., 2010) and *Phyllobacterium* (Mantelin et al., 2006)), Betaproteobacteria (*Herbaspirillum* (Valverde et al., 2003) and *Shinella* (Lin et al., 2008)), Gammaproteobacteria (*Pantoea*, *Enterobacter* and *Pseudomonas* (Benhizia et al., 2004; Ibáñez et al.,

* Corresponding author: Centre for Rhizobium Studies, Murdoch University, Western Australia 6150, Australia. Tel.: +61 893602439; fax: +61 893107084.

E-mail address: Sofdemey@outlook.com (S.E. De Meyer).

Table 1

Genera recovered in this study with their rep-clusters, identification according to partial 16S rRNA gene sequencing and the number of isolates.

Genera	Representative strains	Rep-clusters	# isolates
Actinobacteria			
<i>Actinoplanes</i> sp.	R-45801	Alone	1
<i>Aeromicrobium</i> sp.	R-45950, R-45951	411	13
<i>Arthrobacter</i> sp.	R-45644, R-45645, R-45677, R-45678, R-45679, R-45733, R-46132, R-46278, R-46319	Alone, 317, 403, 443	24
<i>Brevibacterium</i> sp.	R-45585, R-45586	Alone	2
<i>Corynebacterium</i> sp.	R-45865, R-45902, R-45903, R-45927, R-46008	Alone, 172	5
<i>Curtobacterium</i> sp.	R-46162, R-46314	Alone, 69	10
<i>Kocuria</i> sp.	R-45655, R-45665, R-45689, R-45691, R-45692, R-46308	Alone, 257, 442, 448	20
<i>Leifsonia</i> sp.	R-45745, R-46062, R-46076, R-46167, R-46259	Alone, 427	7
<i>Microbacterium</i> sp.	R-45570, R-45573, R-45658, R-45659, R-45676, R-45694, R-45704, R-45758, R-45762, R-45772, R-45841, R-45861, R-45991, R-46024, R-46029, R-46031, R-46041	Alone, 79, 185, 275, 324, 325, 404, 415, 444	43
<i>Microbispora</i> sp.	R-45698	Alone	1
<i>Micromonospora</i> sp.	R-45554	Alone	1
<i>Moraxella</i> sp.	R-45536, R-45904	Alone, 24	10
<i>Mycobacterium</i> sp.	R-45620, R-46056, R-46330	Alone, 77, 180	5
<i>Oerskovia</i> sp.	R-45820	Alone	1
<i>Plantibacter</i> sp.	R-46164	Alone	1
<i>Promicromonospora</i> sp.	R-45862, R-45892, R-46030, R-46035	Alone, 326, 379	16
<i>Rhodococcus</i> sp.	R-45548, R-45551	Alone	2
<i>Sphaerisporangium</i> sp.	R-46174	Alone	1
<i>Streptomyces</i> sp.	R-45560, R-45795, R-45838, R-45839, R-45840, R-45852, R-45853, R-45856, R-45857, R-45858, R-45880, R-46032, R-46033, R-46034, R-46036, R-46037, R-46057, R-46058, R-46156, R-46264, R-46282, R-46320	Alone, 280, 321, 336	30
Alphaproteobacteria			
<i>Ancylobacter</i> sp.	R-45799, R-45800	Alone	2
<i>Bosea</i> sp.	R-45681, R-46060, R-46070, R-46073	Alone, 6	5
<i>Caulobacter</i> sp.	R-46323	Alone	1
<i>Inquilinus</i> sp.	R-45827, R-46318	Alone, 323	3
<i>Novosphingobium</i> sp.	R-45660	329	2
<i>Paracoccus</i> sp.	R-46302, R-46307	Alone	2
<i>Phyllobacterium</i> sp.	R-45564, R-45798, R-46124, R-46157	Alone, 129, 130, 181	9
<i>Sphingomonas</i> sp.	R-46285, R-45731, R-45732	Alone, 211	3
<i>Sphingomonadaceae</i> sp.	R-46192	Alone	1
Betaproteobacteria			
<i>Herbaspirillum</i> sp.	R-45723	Alone	1
<i>Massilia</i> sp.	R-45804, R-45805, R-45830	99, 100, 335	6
<i>Roseateles</i> sp.	R-45571	Alone	1
<i>Variovorax</i> sp.	R-46208	33	10
Firmibacteria			
<i>Bacillus</i> sp.	R-40421, R-45534, R-45535, R-45537, R-45540, R-45543, R-45549, R-45607, R-45608, R-45628, R-45640, R-45650, R-45656, R-45667, R-45669, R-45671, R-45672, R-45706, R-45708, R-45775, R-45785, R-45787, R-45792, R-45793, R-45794, R-45824, R-45833, R-45837, R-45842, R-45851, R-45859, R-45885, R-45890, R-45942, R-45943, R-45944, R-45945, R-45947, R-45997, R-46011, R-46013, R-46020, R-46025, R-46141, R-46144, R-46146, R-46152, R-46169, R-46176, R-46193, R-46216, R-46226, R-46228, R-46238, R-46245, R-46246, R-46263, R-46279, R-46280	Alone, 23, 30, 161, 170, 188, 303, 362, 375, 376, 377, 378, 380, 420, 421, 422, 423, 424, 425, 432, 434, 435, 439, 440	117
<i>Brevibacillus</i> sp.	R-45680	Alone	1
<i>Cohnella</i> sp.	R-45709	428	1
<i>Exigobacterium</i> sp.	R-45918	Alone	1
<i>Lysinibacillus</i> sp.	R-45541, R-45670, R-45748, R-46326	Alone	4
<i>Paenibacillus</i> sp.	R-45550, R-45610, R-45623, R-45647, R-45649, R-45664, R-45673, R-45674, R-45675, R-45701, R-45776, R-45786, R-45807, R-45812, R-45813, R-45814, R-45815, R-45816, R-45939, R-45993, R-46010, R-46038, R-46080, R-46203, R-46243, R-46244, R-46251, R-46252, R-46257, R-46269, R-46305	Alone, 2, 4, 5, 12, 101, 111, 169, 171, 238, 357, 370, 381, 431	84
<i>Staphylococcus</i> sp.	R-45577, R-45580, R-45641, R-45663, R-45688, R-45690, R-45693, R-45875, R-46012, R-46052, R-46142, R-46143	Alone, 7, 406, 412, 413, 419	20
Flavobacteria			
<i>Chryseobacterium</i> sp.	R-45581, R-46064	Alone, 429	7
Gammaproteobacteria			
<i>Acinetobacter</i> sp.	R-45867	313	4
<i>Buttiauxella</i> sp.	R-45774	110	5
<i>Enhydrobacter</i> sp.	R-45682, R-45683, R-45684, R-45685, R-45686, R-45687	Alone, 426, 449	11
<i>Enterobacter</i> sp.	R-45810	82	7
<i>Erwinia</i> sp.	R-45811	Alone	1
<i>Pantoea</i> sp.	R-45539, R-45717, R-45789, R-45806, R-46081, R-46239, R-46301	Alone, 16, 281, 333, 347	27

Table 1 (continued)

Genera	Representative strains	Rep-clusters	# isolates
<i>Pseudomonas</i> sp.	R-45532, R-45533, R-45538, R-45582, R-45716, R-45757, R-45808, R-45809, R-45822, R-45823, R-45850, R-45864, R-45972, R-46026, R-46089, R-46100, R-46111, R-46145, R-46171, R-46240, R-46241, R-46242	Alone, 3, 17, 18, 19, 20, 31, 32, 68, 90, 97, 112, 273, 293	108
<i>Rahnella</i> sp.	R-46079	91	5
<i>Stenotrophomonas</i> sp.	R-46069, R-46300	Alone	2
<i>Xanthomonas</i> sp.	R-45826, R-45893, R-46004, R-46065	Alone, 213	8
Sphingobacteria			
<i>Dyadobacter</i> sp.	R-45763	338	2

2009; Shiraishi et al., 2010; Aserse et al., 2013)), Actinobacteria (*Arthrobacter*, *Microbacterium* and *Curtobacterium* (Sturz et al., 1997; Palaniappan et al., 2010)) and Firmibacteria (*Bacillus*, *Paenibacillus* and *Staphylococcus*, (Rajendran et al., 2008; Palaniappan et al., 2010; Shiraishi et al., 2010; Deng et al., 2011)). They were in most cases isolated on the same yeast mannitol agar medium as rhizobia. The characteristics important for the interaction with legumes seem to be shared by a growing number of unrelated taxa (Sawada et al., 2003).

Certain NRE bacteria have beneficial effects on the host plants, including plant growth promotion (Vessey, 2003; Kuklinsky-Sobral et al., 2004; Ibáñez et al., 2009; El-Tarabily et al., 2010; Tariq et al., 2014), nitrogen fixation (Andrews et al., 2010), siderophore mediated interactions (Rajendran et al., 2008; Andrews et al., 2010), increased promotion of plant stress tolerance (Andrews et al., 2010) and biological control of plant pathogens (El-Tarabily et al., 2010). Most legumes are good pioneer species, adapted to low nutrient soils and/or environments with heavy metals (Gonzalez-Andres et al., 2005; Vidal et al., 2009; Azcon et al., 2010). Multiple studies report on the beneficial effect of co-inoculating rhizobia with other bacteria (Zhang et al., 1996; Parmar and Dadarwal, 1999; Rajendran et al., 2008; Egamberdieva et al., 2010). All previously mentioned studies suggest that besides rhizobia, NRE are present inside root nodules, which may have beneficial effects on the host plant.

While many studies in the past have focussed on plant species of economic importance, including soybean, common bean, cowpea, chickpea and red clover (Delorme et al., 2003; Laranjo et al., 2004; Kuklinsky-Sobral et al., 2005; Duodu et al., 2007; Hung et al., 2007; Laranjo et al., 2008; Ogutcu et al., 2008; Appunu et al., 2009; Chagas et al., 2010; Li et al., 2010; Pule-Meulenberg et al., 2010), native legume species have generally received less attention. In Belgium, legumes are restricted to the *Faboideae* subfamily and contain 102 plant species in 30 different genera (Lambinon et al., 1998). In a previous study, we assessed the genetic diversity of 3810 isolates belonging to traditional rhizobial genera including *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*, isolated from a large diversity of indigenous legumes in Flanders (Belgium) (De Meyer et al., 2011). The present study, is based on the same sampling campaigns, however, it focuses on the other bacteria recovered from those root nodules. Our aim was to analyse the diversity of non-rhizobial root nodule endophytes (NRE) isolated from various indigenous and exotic legume plants in Flanders (Belgium), to gain insights in their potential nitrogen fixation ability and to investigate possible connections with plant species and ecoregion. Additionally, the results on the rhizobia from our previous study (De Meyer et al., 2011) were compared with the diversity found in the present study.

2. Materials and methods

2.1. Nodule collection and isolation

Root nodules were collected as previously reported (De Meyer et al., 2011). Briefly, the sampling campaigns were performed

over the summers of 2008 and 2009. Sampling plots were selected based on the diversity of legume species present (Van Landuyt et al., 2006) and the difference in ecoregion (campine, dune, polder, loamy and sandy-sandloamy region) (Van Landuyt et al., 2006; Van Landuyt et al., 2011). In most cases, whole plants were excavated and taken to the laboratory where the nodules were excised from roots in situ, brushed free of soil debris and preserved at 4 °C in tubes containing dried silica beads. Healthy, non-ruptured nodules were collected to maximise the isolation of root-nodule bacteria. Bacteria were isolated in 2009 and 2010 according to the protocol described previously (De Meyer et al., 2011) and the surface sterilized nodules were rolled over YMA agar plates (Vincent, 1970) to confirm adequate surface sterilisation. The control plates without bacterial growth were considered as successfully surface sterilized and isolates from these nodules were further used in the study. All YMA plates were incubated at 28 °C and regularly checked for growth up to 20 days. Two colonies of each morphological type were selected for isolation. The bacteria were purified by repeatedly streaking on YMA plates. All bacteria were stored in tubes with 15% glycerol and YMA broth at –20 °C.

2.2. DNA extraction and genomic fingerprinting by (GTG)₅ rep-PCR

Total genomic DNA of each isolate was extracted as described by Baele et al. (2000) and a repetitive extragenic palindromic (rep) PCR was performed with the (GTG)₅ primer in a total volume of 25 µl (Versalovic et al., 1994; Gevers et al., 2001). To allow normalization, a combined 500 bp and 100 bp DNA marker (Biorad) was included 4 times in each 1.5% (w/v) agarose gel during electrophoresis (960 min at 55 V (constant voltage)) in a 4 °C incubator. The electrophoresed gels were stained for 30 min in a solution of 1 µg ml^{–1} EtBr in Tris acetic acid EDTA buffer (1 x) and digital pictures were taken under UV light. The rep (GTG)₅ patterns were normalized and cluster analysis was performed using the software package BioNumerics v5.1 (Applied Maths). Representatives of each rep cluster analysed in this study are listed in Table 1 and Table S1.

2.3. Sequence analysis of 16S rRNA gene and ribosomal database project (RDP) classification

The 16S rRNA gene was sequenced using the protocol described previously by Vancanneyt et al. (2004). High quality partial sequences (262–490 bp) were submitted to a FASTA search using the EMBL nucleotide sequence database to find related species or genera (Pearson, 1990). The Ribosomal Database Project (RDP) Classifier, a naïve Bayesian classifier, was used to obtain identifications at genus level (Wang et al., 2007). The RDP classifier estimates the classification reliability using bootstrap confidence estimation values with a default threshold of 80% (Wang et al., 2007). Strains identified with confidence estimates lower than 80% were analysed together with reference strains using the MEGA 5 software package (Tamura et al., 2011). Phylogenetic trees were constructed using the Maximum Likelihood method, with the

General time reversible model. Bootstrap analysis with 500 replicates was performed to assess the reliability of the clustering. Sequences of the 16S rRNA gene determined in this study have been deposited in the EMBL database under the accession numbers: FR774919 to FR775187. Nodulation (*nodC*) and nitrogen fixation (*nifH*) genes have also been investigated according to previously described protocols (De Meyer et al., 2011), however no amplicons were obtained.

2.4. Statistical analysis

All analyses were performed with the statistical computing environment R (R Core Team, 2013), version 3.0.1. A hierarchical cluster analysis based on Ward's method (Ward Jr, 1963) was used to cluster plant species with similar patterns of presence/absence of NRE, given the presence of various rhizobia. Next, a double hierarchical cluster analysis was performed to reveal with which these clusters showed similar co-occurrence patterns of NRE. The results are shown by means of a heatmap. The colours in the heatmap are indicative of the proportion of occurrence of a specific NRE in a certain plant species. More specifically, they are based on the column Z-score of this proportion, which is the normalized proportion within each NRE in this case. Two additional double hierarchical cluster analyses were performed to reveal ecoregions with similar co-occurrence patterns of NRE and to reveal rhizobia with similar co-occurrence patterns of NRE. Heatmaps were constructed as described above.

To assess the sample coverage of NRE and rhizobia in our study, several rarefaction curves were calculated using the online rarefaction calculator (<http://biome.sdsu.edu/fastgroup/caltools.htm>). Different intensively sampled plant species, including *Trifolium pratense*, *Trifolium repens*, *Medicago lupulina* and *Vicia cracca* and two less intensively sampled plant species harbouring a large diversity of bacteria in their root nodules were analysed. Similarly, rarefaction curves were calculated for each ecoregion sampled in this study.

3. Results

3.1. Sampling and isolation of bacteria

We obtained 654 bacterial isolates originating from 162 surface sterilised root nodules from 30 plant species in 14 genera (Table S1). These isolates were obtained as part of a larger sampling campaign covering different regions in Flanders (De Meyer et al., 2011): one or two representatives from each legume species present in selected sampling sites were excavated and nodules collected. One nodule per sampled plant was selected and used for isolation. The surface sterilization treatment was generally efficient, since in most cases no growth occurred on the YMA plates upon which the surface sterilized nodules were rolled. All colony types appearing on the YMA plates were purified and investigated. There was a large variation in colony morphology; differences were observed in colour, shape and size (data not shown). In total, 4464 isolates were obtained (De Meyer et al., 2011). Here we report on the bacteria not belonging to the traditional rhizobia genera. They were recovered from all reported plant genera except for *Galega*, *Securigera* and *Ulex*. Our previous report covered the 3810 isolates that were found to be traditional rhizobia (De Meyer et al., 2011).

The rarefaction curves indicate that only for *T. pratense* and *T. repens* host plants the diversity was covered since these curves are reaching a plateau (Fig. S1). For all other plant species rarefaction curves are rising indicating that the diversity was not yet covered completely. Only the rarefaction curve for the Loamy ecoregion reaches a plateau and as a consequence covers fairly well the

bacterial diversity in that region (Fig. S2). For all other ecoregions, graphs are rising and additional samples should be investigated to fully cover the bacterial diversity.

3.2. Rep (GTG)₅ PCR fingerprinting

High resolution fingerprinting patterns were obtained in the (GTG)₅-PCR analysis which allowed identification of duplicate isolates, to reduce the number of strains and to get a first insight on the extent of diversity sampled. The reproducibility of this technique was analysed by including one control strain in each PCR. This resulted in repeated fingerprinting patterns with correlation levels of 90,8% to 98,3%, in line with previously reported data (Gevers et al., 2001) and confirming the strength of this technique. A Pearson correlation/UPGMA analysis was performed and rep-clusters were delineated at 80% correlation. This revealed a high level of diversity with 106 rep-clusters and 155 strains occupying isolated positions (Table S1). With regard to the number of colonies selected for purification, two of each morphology type seems reasonable since these duplicates always grouped within the same rep-cluster. In total, 269 strains were selected based on their unique rep-profile for further genetic identification.

3.3. Phylogenetic analysis of 16S rRNA

The RDP classifications and phylogenetic trees obtained with the 16S rRNA gene sequences revealed an enormous diversity including different taxa in the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmibacteria, Flavobacteria and Sphingobacteria (Fig. S3). The genera recovered with their representative strains and rep-clusters are summarised in Table 1 and detailed information on representative strains is listed in Supplementary Table S1. Actinobacteria were related to *Actinoplanes*, *Aeromicrobium*, *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Kocuria*, *Leifsonia*, *Microbacterium*, *Moraxella*, *Mycobacterium*, *Oerskovia*, *Plantibacter*, *Promicromonospora*, *Rhodococcus*, *Schumanella* and *Streptomyces* (Fig. S4). The Firmibacteria were related to *Bacillus*, *Brevibacillus*, *Cohnella*, *Exigobacterium*, *Lysinibacillus*, *Paenibacillus* and *Staphylococcus* (Fig. S5). Alphaproteobacteria strains were related to *Ancylobacter*, *Bosea*, *Caulobacter*, *Inquilinus*, *Novosphingobium*, *Paracoccus*, *Phyllobacterium* and *Sphingomonas* (Fig. S6). The Betaproteobacteria strains were related to *Herbaspirillum*, *Massilia*, *Roseateles* and *Variovorax* (Fig. S6). Gammaproteobacteria strains were related to *Acinetobacter*, *Buttiauxella*, *Enhydrobacter*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Rahnella*, *Stenotrophomonas* and *Xanthomonas* (Fig. S6).

Overall, Firmibacteria (34.9%) were the most abundantly recovered, followed by Actinobacteria (29.5%), Gammaproteobacteria (27.2%), Alphaproteobacteria (4.3%) and Betaproteobacteria (2.8%) (Table 1, Fig. S3). A limited number of isolates were related to Flavobacteria (1%) and Sphingobacteria (0.3%). *Bacillus* (17.9%) and *Pseudomonas* (15.9%) were the most frequently recovered bacterial genera within this NRE population.

3.4. Host plant – NRE association

The legumes sampled were identified as 30 species divided over 14 genera: *Anthyllis*, *Colutea*, *Cytisus*, *Lathyrus*, *Lotus*, *Lupinus*, *Medicago*, *Melilotus*, *Ononis*, *Ornithopus*, *Robinia*, *Trifolium*, *Vicia* and *Wisteria*. An overview of the host plant species with their NRE is shown in Table 2.

Double cluster analysis was performed to reveal plants with similar co-occurrence patterns of NRE (Fig. 1). These data group the host plants in six clusters according to the presence of particular NRE species. Cluster 1 contains *Cytisus scoparius*, *Lathyrus pratensis*,

Table 2Host plants with the number of non-rhizobial endophytes found per plant species.^a

Plant genera	Cytisus	Lathyrus			Lotus		Lupinus	Medicago		Melilotus			Ononis	Ornithopus	Robinia	Trifolium					Vicia		
Plant species ^b	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Actinobacteria																							
<i>Actinoplanes</i> sp.	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Aeromicrobium</i> sp.	—	—	—	—	—	12	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
<i>Arthrobacter</i> sp.	—	—	—	—	1	—	11	—	—	—	—	5	—	—	4	—	—	—	—	2	—	—	1
<i>Brevibacterium</i> sp.	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Corynebacterium</i> sp.	—	—	—	—	1	—	—	2	—	—	—	—	—	—	—	—	1	1	—	—	—	—	—
<i>Curtobacterium</i> sp.	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	4	—	3	—	1	—	—
<i>Kocuria</i> sp.	1	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	14	—
<i>Leifsonia</i> sp.	1	—	—	—	—	—	—	—	—	—	—	—	—	1	2	—	—	—	—	—	3	—	—
<i>Microbacterium</i> sp.	2	14	—	—	—	—	12	4	—	1	—	—	1	6	—	2	—	—	—	1	—	—	—
<i>Microbispora</i> sp.	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Micromonospora</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—
<i>Moraxella</i> sp.	1	—	1	—	—	—	—	2	2	1	—	1	—	—	1	—	—	—	—	—	1	—	—
<i>Mycobacterium</i> sp.	—	2	1	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Plantibacter</i> sp.	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Promicromonospora</i> sp.	—	13	—	—	—	—	—	1	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—
<i>Rhodococcus</i> sp.	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Sphaerisporangium</i> sp.	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Streptomyces</i> sp.	1	15	1	—	—	—	—	—	3	—	1	1	3	—	2	2	—	—	—	1	—	—	—
Alphaproteobacteria																							
<i>Ancylobacter</i> sp.	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Bosea</i> sp.	—	2	—	—	—	—	1	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—
<i>Caulobacter</i> sp.	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Inquilinus</i> sp.	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Novosphingobium</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	2	—	—	—
<i>Paracoccus</i> sp.	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Phyllobacterium</i> sp.	5	—	—	—	3	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
<i>Sphingomonas</i> sp.	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Xanthobacteraceae</i> sp.	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Betaproteobacteria																							
<i>Herbaspirillum</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—
<i>Massilia</i> sp.	—	—	—	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Roseateles</i> sp.	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Variovorax</i> sp.	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	4	—	1	—	—	—	3
Firmibacteria																							
<i>Bacillus</i> sp.	13	—	5	—	6	20	14	20	1	4	2	8	1	1	—	1	—	2	1	—	6	5	—
<i>Brevibacillus</i> sp.	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Cohnella</i> sp.	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Exigobacterium</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
<i>Lysinibacillus</i> sp.	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
<i>Paenibacillus</i> sp.	8	—	2	2	—	18	6	—	—	8	—	—	—	1	8	—	—	5	5	5	5	9	2
<i>Staphylococcus</i> sp.	—	—	1	—	2	6	5	3	—	1	—	—	—	—	—	—	—	—	—	—	1	—	—
Flavobacteria																							
<i>Chryseobacterium</i> sp.	1	—	—	—	—	5	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
Gammaproteobacteria																							
<i>Acinetobacter</i> sp.	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Enhydrobacter</i> sp.	—	—	—	—	—	—	11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Enterobacter</i> sp.	—	—	—	3	—	—	—	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Erwinia</i> sp.	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Pantoea</i> sp.	—	—	5	—	—	1	—	1	—	—	—	1	—	—	12	5	—	—	—	—	—	2	—
<i>Pseudomonas</i> sp.	—	7	6	4	17	7	1	11	—	8	—	4	—	—	20	2	—	—	2	4	14	—	—
<i>Rahnella</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—
<i>Stenotrophomonas</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1	—
<i>Xanthomonas</i> sp.	—	—	—	—	1	2	—	—	—	—	—	—	—	—	3	—	—	—	2	—	—	—	—
Sphingobacteria																							
<i>Dyadobacter</i> sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—

^a Plant species that were sampled less than three times and that harbour only one endosymbiont are not included in the table, but mentioned hereafter. *Anthyllis vulneraria*: *Bacillus* sp.(3), *Colutea arborescens*: *Buttiauxella* sp.(5), *Medicago falcata*: *Paracoccus* sp.(1), *Melilotus indicus*: *Bacillus* sp.(1), *Vicia lathyroides*: *Lysinibacillus* sp.(1), *Vicia sepium*: *Pseudomonas* sp.(1), *Vicia tetrasperma*: *Lysinibacillus* sp.(1), *Wisteria sinensis*: *Bacillus* sp.(3).

^b Plant species: 1, *Cytisus scoparius*; 2, *Lathyrus latifolius*; 3, *Lathyrus pratensis*; 4, *Lathyrus sylvestris*; 5, *Lotus corniculatus*; 6, *Lotus pedunculatus*; 7, *Lupinus polyphyllus*; 8, *Medicago lupulina*; 9, *Medicago sativa*; 10, *Melilotus albus*; 11, *Melilotus altissimus*; 12, *Melilotus officinalis*; 13, *Ononis repens*; 14, *Ornithopus perpusillus*; 15, *Robinia pseudoacacia*; 16, *Trifolium arvense*; 17, *Trifolium dubium*; 18, *Trifolium hybridum*; 19, *Trifolium pratense*; 20, *Trifolium repens*; 21 *Vicia cracca*; 22, *Vicia hirsuta*; 23, *Vicia sativa*.

Trifolium arvense, *Trifolium hybridum*, *Vicia cracca*, *Vicia hirsuta* and *Wisteria sinensis* host plants and is characterised by the dominance of *Streptomyces* and *Rhodococcus* bacteria. Cluster 2 contains *Lotus corniculatus*, *Lotus pedunculatus*, *Medicago sativa*, *Melilotus officinalis*, *Ononis repens*, *Trifolium pratense*, *Trifolium repens* and *Vicia sativa* host plants and has a dominance of *Xanthomonas*, *Variovorax*, *Promicromonospora*, *Inquilinus* and *Novosphingobium* bacteria.

Cluster 3 only consists of *Lupinus polyphyllus* and has *Staphylococcus*, *Microbacterium*, *Kocuria*, *Brevibacillus* and *Enhydrobacter* as dominant NRE. Cluster 4 contains *Lathyrus latifolius*, *Lathyrus sylvestris*, *Medicago lupulina*, *Melilotus alba* and *Vicia sepium* host plants and is characterised by the dominance of *Enterobacter*, *Brevibacterium* and *Paenibacillus* NRE. Cluster 5 only consists of *Ornithopus perpusillus* and has a dominance of *Leifsonia*, *Herbaspirillum*

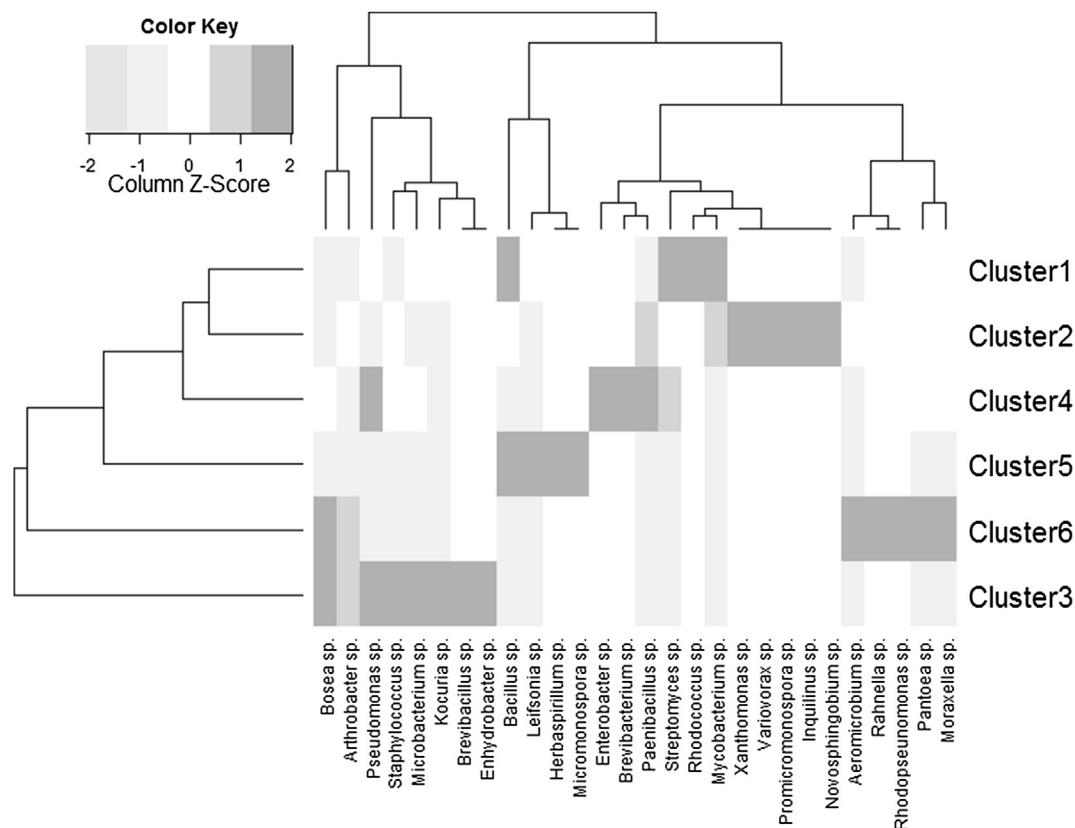


Fig. 1. Double cluster analysis of host plants with their NRE. Cluster 1: *Cytisus scoparius*, *Lathyrus pratensis*, *Trifolium arvense*, *Trifolium hybridum*, *Vicia cracca*, *Vicia hirsuta* and *Wisteria sinensis*; Cluster 2: *Lotus corniculatus*, *Lotus pedunculatus*, *Medicago sativa*, *Melilotus officinalis*, *Ononis repens*, *Trifolium pratense*, *Trifolium repens* and *Vicia sativa*; Cluster 3: *Lupinus polyphyllus*; Cluster 4: *Lathyrus latifolius*, *Lathyrus sylvestris*, *Medicago lupulina*, *Melilotus alba* and *Vicia sepium*; Cluster 5: *Ornithopus perpusillus* and Cluster 6: *Robinia pseudoacacia*. NRE that appear less than two times are not included and plants in which no NRE were found are not included.

and *Micromonospora* bacteria. Finally, cluster 6 contains only *Robinia pseudoacacia* as host plant and is characterised by the dominance of *Rahnella*, *Aeromicrobium*, *Pantoea* and *Moraxella* species.

Of the plant genus *Cytisus*, only the species *Cytisus scoparius* was sampled and its NRE were dominated by *Bacillus* (34%) and *Paenibacillus* (21%) species, accounting for more than 50% of all NRE found in this plants species. All other isolated bacterial species were recovered in less prominent amounts (<13%). The genus *Lathyrus* was represented by *Lathyrus latifolius*, *Lathyrus pratensis* and *Lathyrus sylvestris*. Remarkably, each of the *Lathyrus* species harboured different NRE in their root nodules, in addition to *Pseudomonas* species accounting for respectively 13%, 27% and 40% of the total NRE isolates. *Lathyrus latifolius* root nodule samples were dominated by *Streptomyces* (28%), *Microbacterium* (26%) and *Promicromonospora* (25%) species. The *Lathyrus pratensis* endophytes were dominated by *Pseudomonas* (27%) and *Bacillus* (23%) species. Finally, *Lathyrus sylvestris* contained a limited diversity of NRE mostly represented by *Pseudomonas* (40%), *Enterobacter* (30%) and *Bacillus* (20%) species. The *Lotus* species investigated in this study, *Lotus corniculatus* and *Lotus pedunculatus*, shared in common the following NRE: *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Xanthomonas*, although relative abundances differ in both plant species (Table 2). Within the genus *Lupinus*, only one species was sampled, *Lupinus polyphyllus*, and the predominant NRE isolated were *Bacillus* (20%) and *Microbacterium* (17%) species. Between *Medicago lupulina* and *Medicago sativa*, large differences were found in the presence of NRE, however both plant species were not equally sampled, as was also the case for the *Melilotus* plant species. Nevertheless, they all harboured *Bacillus* species in their root

nodules. Plant genera *Ononis* and *Ornithopus* are both represented in this study by a single species, *Ononis repens* and *Ornithopus perpusillus*, and they harboured respectively *Streptomyces* (43%) and *Microbacterium* (46%) as dominant NRE. *Robinia pseudoacacia*, is a remarkably promiscuous host harbouring 13 different NRE genera. More than 50% belonged to the Gammaproteobacteria genera *Pantoea* and *Pseudomonas*. Furthermore, five Actinobacteria genera were present accounting for 16% of the isolates. Five *Trifolium* species were analysed in this study, *Trifolium arvense*, *Trifolium dubium*, *Trifolium hybridum*, *T. pratense* and *T. repens*. In three of the five plant species *Bacillus*, *Paenibacillus* and *Pseudomonas* NRE were present; all other bacteria differ greatly in presence and abundance (Table 2). The *Vicia* plant species investigated in this study include *V. cracca*, *Vicia hirsuta*, *Vicia lathyroides*, *V. sativa*, *V. sepium* and *Vicia tetrasperma*. *Paenibacillus* was the dominant NRE in half of these plant species (Table 2). However, not all *Vicia* species were sampled equally.

3.5. Ecoregion – NRE association

As previously described, plants were collected from different ecoregions in Flanders (De Meyer et al., 2011). These ecoregions are based on climatologic conditions, geology, geomorphology, soil type, groundwater and surface water content (Van Landuyt et al., 2006) and thus reflects soil texture, water content and nutrient availability. Double cluster analysis on the presence of NRE in certain ecoregions was performed using Ward's method in R and the results are given in a heatmap (Fig. 2). For each ecoregion characteristic patterns of present and/or absent NRE genera is

visualised. *Paenibacillus*, *Kocuria* and *Leifsonia* NRE dominate the Campine ecoregion. The Dunes ecoregions is characterized by the dominance of *Promicromonospora*, *Moraxella*, *Aeromicrobium*, *Microbacterium*, *Rahnella*, *Bosea* and *Rhodopseudomonas* NRE dominate the Loamy ecoregions, whereas *Xanthomonas*, *Inquilinus*, *Enterobacter*, *Pantoea* and *Streptomyces* dominate the Polders ecoregions. The Sandy and sandloamy ecoregion is characterized by a dominance of *Brevibacterium*, *Novosphingobium*, *Variovorax*, *Brevibacillus*, *Enhydrobacter*, *Mycobacterium*, *Micromonospora*, *Rhodococcus*, *Herbaspirillum*, *Staphylococcus* and *Arthrobacter* NRE.

3.6. Rhizobia – NRE association

When combining the data obtained from our previous study (De Meyer et al., 2011) and the current study we see that on average across plant species 84.1% of the isolates were traditional rhizobia (*Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*) ranging between 77.7% and 90.5% at a confidence interval of 95%. A detailed comparison of the investigated host plants shows that for the majority of the plant species far more rhizobia than NRE were recovered from the nodules (Fig. 3). However, *Anthyllus vulneraria* and *Medicago falcata* yielded a 50% ratio of both. In general, plant genera *Trifolium* and *Vicia* seem to be restrictive hosts with low numbers of NRE (4%), whereas *Lathyrus* and *Lotus* genera yielded a larger number of NRE (41% and 31%, respectively see Fig. 3). Ratio analysis of rhizobia versus NRE in the different ecoregions revealed 75.87% rhizobia in the Campine ecoregion, 76.96% in the Dunes ecoregion, 87.17% in the Polders ecoregion, 88.53% in the Sandy and sandloamy ecoregion and 91.96% in the Loamy ecoregion. Additionally, a double cluster analysis was performed for rhizobia and NRE, to understand the correlation between the two groups. The results are shown in a heat map (Fig. 4) and highlight the

correlation of rhizobia species occurrence with certain NRE in root nodules. Within the genus *Bradyrhizobium* we found a co-occurrence of *B. canariense* with *Bacillus*, *Rhodococcus* and *Streptomyces* and *Bradyrhizobium japonicum* with *Pantoea*, *Bacillus*, *Streptomyces*, *Staphylococcus* and *Arthrobacter*. All *Ensifer* species had *Pseudomonas* and *Moraxella* as dominant NRE. Additionally, *E. kummerowiae* had *Mycobacterium*, *Brevibacterium*, *Staphylococcus* and *Paenibacillus* as NRE. *E. meliloti* co-occurred with *Pantoea*, *Bacillus* and *Paenibacillus*, whereas *E. medicae* only had *Pantoea* as additional NRE. Within the genus *Mesorhizobium* we found co-occurrence of *Mesorhizobium loti* with *Rahnella*, *Aeromicrobium*, *Bosea*, *Pantoea*, *Moraxella* and *Rhodopseudomonas*. For *Rhizobium*, correlations were found for *Rhizobium giardinii* with *Staphylococcus*, *Inquilinus* and *Arthrobacter*, for *Rhizobium cellulosilyticum* and *Rhizobium radiobacter* with *Enterobacter*, *Pantoea* and *Pseudomonas*, for *Rhizobium alamii* with *Microbacterium* and *Moraxella*, and for *Rhizobium leguminosarum* with *Bacillus*, *Moraxella*, *Pseudomonas* and *Pantoea*. Given that rarefaction curves for most host species indicate not all diversity was covered, further studies are needed to assess the significance of these observations.

4. Discussion

Previously, we reported on traditional rhizobia from native legume species in Flanders that were collected over the summers of 2008 and 2009 (De Meyer et al., 2011). In addition to 3810 rhizobia isolates characterised previously (De Meyer et al., 2011), these campaigns also yielded 654 NRE that were studied using 16S rRNA, *nodC* and *nifH* gene sequencing. Only nodules that appeared healthy (presence of red colour) were harvested and analysed further.

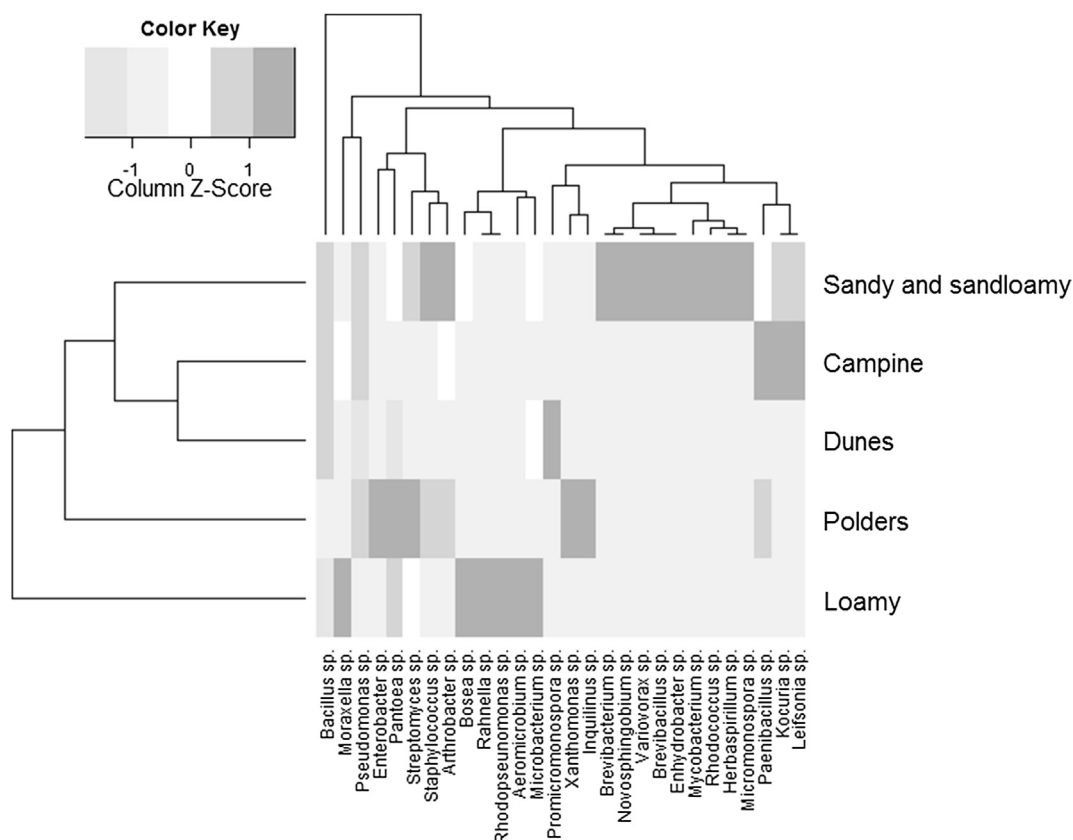


Fig. 2. Double cluster analysis of the ecoregions with their NRE. NRE that appear less than two times are not included.

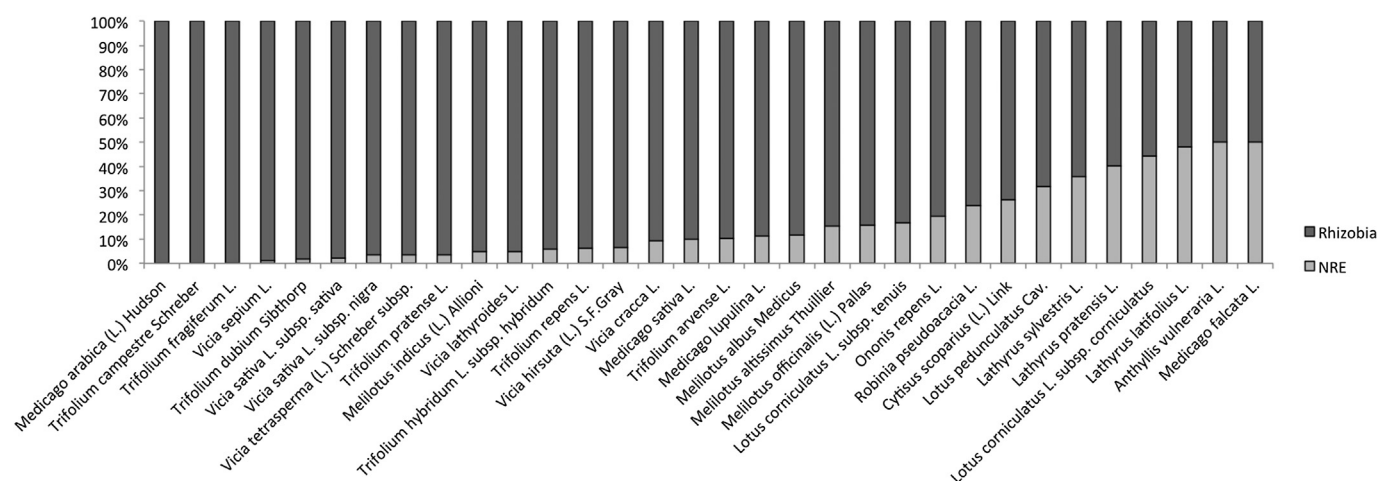


Fig. 3. Bar chart representing the percentage rhizobia and NRE recovered from the different host plants.

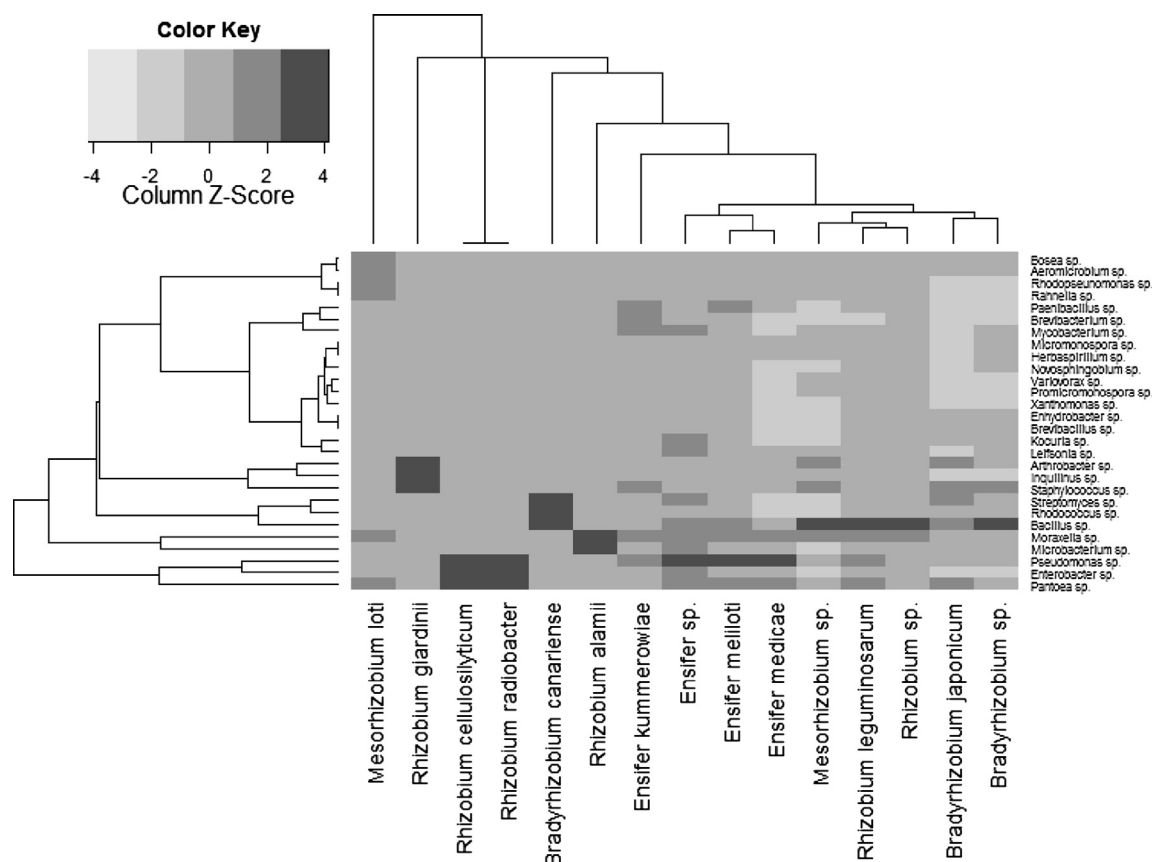


Fig. 4. Double cluster analysis of rhizobia and NRE. Bacteria that appear less than two times are not included.

The data presented in this study suggest the presence of a large diversity of bacteria belonging to 50 genera inside nodules of indigenous legumes in Flanders. Plants house several endophytes within their tissues and multiple studies have reported the presence of endophytes in legumes (Muresu et al., 2008; Ibáñez et al., 2009; Muresu et al., 2010). One of the genera in our study, *Phyllobacterium* (Table 1) is a known nodule endosymbiont and is capable of fixing nitrogen (Valverde et al., 2005; Baimiev et al., 2007). However, in our study no symbiosis or nitrogen fixation related genes were detected for the isolates of this genus. Some genera

including, *Arthrobacter*, *Microbacterium*, *Rhodococcus*, *Sphingomonas*, *Bacillus*, *Cohnella*, *Pseudomonas* and *Herbaspirillum*, were previously reported from root nodules of several legume species (Sturz et al., 1997; Mantelin et al., 2006; Zakhia et al., 2006; Putnam and Miller, 2007; Zurdo-Pineiro et al., 2007; Palaniappan et al., 2010). Other bacterial genera encountered, including *Pantoea*, *Corynebacterium*, *Chryseobacterium*, *Sphingomonas* and *Xanthomonas*, were described previously as endophytes in certain plant tissues including legume plant tissue (Burch and Sarathchandra, 2006). However, 33 of the 50 bacterial genera found in this study were to

our knowledge, not previously reported from legume root nodules (Table 1). The majority of the investigated nodules contained *Bacillus* (17.9%), *Paenibacillus* (12.5%) and *Pseudomonas* (15.9%) NRE species, together with small numbers of other NRE bacterial genera from the classes Actinobacteria, Alpha- and Betaproteobacteria.

Nodulation and nitrogen fixation capacity are characters usually studied in rhizobia research, since they give an indication of the host specificity and nitrogen fixing ability (Perret et al., 2000; Moulin et al., 2004; Diouf et al., 2010). No *nodC* or *nifH* gene sequences could be detected among the isolates, indicating an endophytic lifestyle rather than a rhizobial lifestyle. However, this needs to be confirmed using authentication experiments (Howieson et al., 1995). Previous studies indicate that endophytes can have three main functions inside root nodules, firstly as true rhizobial endosymbionts capable of inducing nodulation and performing nitrogen fixation (Valverde et al., 2005; Ardley et al., 2012). Secondly, as helper bacteria increasing plant health and/or yield when co-inoculated with rhizobia (Annapurna et al., 2013; Tariq et al., 2014), and finally, as opportunistic endophytes thriving in this nitrogen rich nodule environment (Dudeja et al., 2012). Therefore, nodules seem to be an excellent environment for a wide variety of bacteria.

The presence of the NRE was analysed in function of the host plant and this indicates that certain plant groups prefer certain NRE in their root nodules (Fig. 1). However, these observations need to be confirmed, given that rarefaction curves for most host species were still rising slightly (Fig. S1). As Dudeja et al. (2012) mentioned, most endophytes originate from the rhizosphere or phyllosphere and only a small number may be transmitted via seed. We postulate that the host plant may select the appropriate NRE from the environment and hence characteristic NRE for certain legume species were found. Interestingly *Trifolium* and *Viciae* plant species harboured a small diversity of bacteria in their root nodules, with a dominance of *Rhizobium* species and more specific *R. leguminosarum*. If NRE can enter the root nodule as opportunists then *Rhizobium* bacteria might have defence mechanisms in place to prevent this from happening, as previously shown against arbuscular mycorrhizal fungi (Franzini et al., 2013) and against soil bacterial populations (Robledo et al., 1998; Wilson et al., 1998). Alternatively, the native *Rhizobium* strains could be highly competitive root nodule bacteria, quickly colonising the available root nodule space and therefore preventing other bacteria from getting established. This has been shown previously in studies where commercial inoculum strains were applied and out competed by native *Rhizobium* strains (Nangul et al., 2013). Remarkable is also the tentative correlation we found between certain NRE and traditional rhizobia. Previous studies have found co-occurrence of *Bacillus* species together with *Bradyrhizobium japonicum* in soybean nodules (Bai et al., 2002) and with *M. gobiense* in *Sphaerophysa salsula* root nodules (Deng et al., 2011). *Paenibacillus* and *Pseudomonas* species have also been found to co-occur with *Mesorhizobium* species (Deng et al., 2011). However, our study reveals an even more complex interaction between traditional rhizobia and NRE bacteria for legumes growing in the temperate climate in Western Europe. Most of the plants harboured *Bacillus* and *Pseudomonas* species in their root nodules together with traditional rhizobia, which is becoming a common observation (Dudeja et al., 2012). The function of these NRE as helpers still needs to be confirmed but the fact that these specific co-occurrence patterns exist, might suggest that not only the plant selects certain NRE but also the rhizobia inside the root nodules prefer certain NRE.

The NRE were also analysed in function of the biogeographical ecoregion they were isolated from. Flanders, the northern part of Belgium, is situated in the Atlantic biogeographical region and has limited climate variation (Van Landuyt et al., 2011). Geological and

landscape elements, such as soil type and land use system are the driving forces in the small-scale distribution of plant species. Several characteristics, including soil type, landscape morphology, land-use system, climate, topography and hydrology were used by Van Landuyt et al. (2006) to designate six ecoregions (campine, dunes, loamy, polders, sandy-sandloamy region and the region of the Valley of the River Meuse) as areas with a more or less uniform landscape. A double cluster analysis was performed using Ward's method in R and revealed a correlation between NRE species and the ecoregion where the plants were collected (Fig. 2). Noticeable is also the fact that ecoregions with similar environmental factors have more similar NRE, as is the case for the Dunes and Campine ecoregions (Fig. 2). The significance of these observations remains to be confirmed given that rarefaction curves were still rising. However, such biogeographical correlation of NRE has been reported before and our study confirms these findings (Deng et al., 2011). However, the NRE groups from the plant analysis are not the same as those in the ecoregion analysis, indicating that different environmental parameters drive the selection of NRE. It is not clear at this point what these factors might be. However, firstly Flanders is a densely populated area and also serves as crossroads for goods and people in Western Europe. Secondly, most of the sampled legumes occur everywhere in Flanders and have thus no specific niche with respect to the ecoregion. Both considerations may contribute to the limited correlation of host plants with ecoregions and available NRE. Small-scale differences in ecoregions may affect bacterial distribution by enabling speciation, permitting certain NRE adapted to these conditions to thrive in these environments and as such explaining the preference of certain NRE for certain ecoregions.

In conclusion, this study investigated a large diversity of legume plant species and their associated NRE. The detection of bacteria other than traditional rhizobia in legume plants has been reported before, however, to our knowledge, this is the first report of such an extensive bacterial diversity analysis in root nodules of indigenous legumes in Western Europe.

Acknowledgements

We thank the Special Research Fund (BOF)-UGent and the Foundation for Scientific Research (FWO)-Flanders for research grants 01J04807 (BOF) and G.0058.07N (FWO). We thank the Research Institute for Nature and Forest (INBO) and the Natuurpunt organisation for facilitating access to nature reserves in Flanders. We are grateful to Prof. Paul Goetghebeur for confirming the plant determinations and the use of the Botanical Garden of the university, to Wouter Van Landuyt for supplying the INBO database and the ecoregion data.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2015.01.002>.

References

- Andrews, M., Hodge, S., Raven, J., 2010. Positive plant microbial interactions. *Annals of Applied Biology* 317–320.
- Annapurna, K., Ramadoss, D., Bose, P., VithalKumar, L., 2013. In situ localization of *Paenibacillus polymyxa* HKA-15 in roots and root nodules of soybean (*Glycine max* L.). *Plant and Soil* 373, 641–648.
- Appunu, C., Sasirekha, N., Prabavathy, V.R., Nair, S., 2009. A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly belong to *Bradyrhizobium* and *Ensifer* genera. *Biology and Fertility of Soils* 46, 57–63.
- Ardley, J., Parker, M.A., De Meyer, S.E., O'Hara, G., Reeve, W., Yates, R.J., Dilworth, M., Willems, A., Howieson, J., 2012. *Microvirga lupini* sp. nov., *Microvirga lotonoidis*

- sp. nov., and *Microvirga zambiensis* sp. nov. are alphaproteobacterial root nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. *International Journal of Systematic and Evolutionary Microbiology* 62, 2579–2588.
- Aserse, A.A., Rasanen, L.A., Aseffa, F., Hailemariam, A., Lindstrom, K., 2013. Diversity of sporadic symbionts and nonsymbiotic endophytic bacteria isolated from nodules of woody, shrub, and food legumes in Ethiopia. *Applied Microbiology and Biotechnology* 97, 10117–10134.
- Azcon, R., Peralvarez, M.D., Roldan, A., Barea, J.M., 2010. Arbuscular mycorrhizal fungi, *Bacillus cereus*, and *Candida parapsilosis* from a multicontaminated soil alleviate metal toxicity in plants. *Microbial Ecology* 59, 668–677.
- Baele, M., Baele, P., Vaneechoutte, M., Storms, V., Butaye, P., Devriese, L.A., Verschraegen, G., Gillis, M., Haesebrouck, F., 2000. Application of rRNA intergenic spacer PCR for identification of *Enterococcus* species. *Journal of Clinical Microbiology* 38, 4201–4207.
- Bai, Y.M., D'Aoust, F., Smith, D.L., Driscoll, B.T., 2002. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. *Canadian Journal of Microbiology* 48, 230–238.
- Baimiev, A.K., Gubaidullin II, Kulikova, O.L., Chmeris, A.V., 2007. Bacteria closely related to *Phyllobacterium trifolii* according to their 16S rRNA gene are discovered in the nodules of Hungarian sainfoin. *Russian Journal of Genetics* 43, 587–590.
- Bautista, V.V., Monsalud, R.G., Yokota, A., 2010. *Devosia yakushimensis* sp. nov., isolated from root nodules of *Pueraria lobata* (Willd.) Ohwi. *International Journal of Systematic and Evolutionary Microbiology* 60, 627–632.
- Benhizia, Y., Benhizia, H., Benguedouar, A., Muresu, R., Giacomini, A., Squartini, A., 2004. Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. *Systematic and Applied Microbiology* 27, 462–468.
- Burch, G., Sarathchandra, U., 2006. Activities and survival of endophytic bacteria in white clover (*Trifolium repens* L.). *Canadian Journal of Microbiology* 52, 848–856.
- Chagas, A.F., de Oliveira, L.A., de Oliveira, A.N., 2010. Phenotypic characterization of rhizobia strains isolated from Amazonian soils and symbiotic efficiency in cowpea. *Acta Scientiarum-Agronomy* 32, 161–169.
- Chen, W.M., Laevens, S., Lee, T.M., Coenye, T., De Vos, P., Mergeay, M., Vandamme, P., 2001. *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *International Journal of Systematic and Evolutionary Microbiology* 51, 1729–1735.
- De Meyer, S.E., Cnockaert, M., Ardley, J.K., Maker, G., Yates, R., Howieson, J.G., Vandamme, P., 2013a. *Burkholderia sprentiae* sp. nov., isolated from *Lebeckia ambigua* root nodules. *International Journal of Systematic and Evolutionary Microbiology* 63, 3950–3957.
- De Meyer, S.E., Cnockaert, M., Ardley, J.K., Trengove, R.D., Garau, G., Howieson, J.G., Vandamme, P., 2013b. *Burkholderia rhynchosiae* sp. nov., isolated from *Rhynchosia ferulifolia* root nodules. *International Journal of Systematic and Evolutionary Microbiology* 63, 3944–3949.
- De Meyer, S.E., Cnockaert, M., Ardley, J.K., Van Wyk, B.-E., Vandamme, P.A., Howieson, J.G., 2014. *Burkholderia diiworthii* sp. nov., isolated from *Lebeckia ambigua* root nodules. *International Journal of Systematic and Evolutionary Microbiology* 64, 1090–1095.
- De Meyer, S.E., Van Hoorde, K., Vekeman, B., Braeckman, T., Willems, A., 2011. Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). *Soil Biology & Biochemistry* 43, 2384–2396.
- Delorme, T.A., Gagliardi, J.V., Angle, J.S., van Berkum, P., Chaney, R.L., 2003. Phenotypic and genetic diversity of rhizobia isolated from nodules of clover grown in a zinc and cadmium contaminated soil. *Soil Science Society of America Journal* 67, 1746–1754.
- Deng, Z.S., Zhao, L.F., Kong, Z.Y., Yang, W.Q., Lindström, K., Wang, E.T., Wei, G.H., 2011. Diversity of endophytic bacteria within nodules of the *Sphaerophysa salula* in different regions of Loess Plateau in China. *FEMS Microbiology Ecology* 76, 463–475.
- Diouf, D., Fall, D., Chantreuil, C., Ba, A.T., Dreyfus, B., Neyra, M., Ndoeye, I., Moulin, L., 2010. Phylogenetic analyses of symbiotic genes and characterization of functional traits of *Mesorhizobium* spp. strains associated with the promiscuous species *Acacia seyal* Del. *Journal of Applied Microbiology* 108, 818–830.
- Dudeja, S.S., Giri, R., Saini, R., Suneja-Madan, P., Kothe, E., 2012. Interaction of endophytic microbes with legumes. *Journal of Basic Microbiology* 52, 248–260.
- Duodu, S., Carlsson, G., Huss-Danell, K., Svenning, M.M., 2007. Large genotypic variation but small variation in N-2 fixation among rhizobia nodulating red clover in soils of northern Scandinavia. *Journal of Applied Microbiology* 102, 1625–1635.
- Egamberdieva, D., Berg, G., Lindström, K., Räsänen, L.A., 2010. Co-inoculation of *Pseudomonas* spp. with *Rhizobium* improves growth and symbiotic performance of fodder galega (*Galega orientalis* Lam. *European Journal of Soil Biology* 46, 269–272.
- El-Tarabily, K.A., Hardy, G.E.S., Sivasithamparan, K., 2010. Performance of three endophytic actinomycetes in relation to plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber under commercial field production conditions in the United Arab Emirates. *European Journal of Plant Pathology* 128, 527–539.
- Estrella, M.J., Munoz, S., Soto, M.J., Ruiz, O., Sanjuan, J., 2009. Genetic diversity and host range of rhizobia nodulating *Lotus tenuis* in typical soils of the Salado river basin (Argentina). *Applied and Environmental Microbiology* 75, 1088–1098.
- Franzini, V.I., Azcon, R., Mendes, F.L., Aroca, R., 2013. Different interaction among *Glomus* and *Rhizobium* species on *Phaseolus vulgaris* and *Zea mays* plant growth, physiology and symbiotic development under moderate drought stress conditions. *Plant Growth Regulation* 70, 265–273.
- Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiology Letters* 205, 31–36.
- Gonzalez-Andres, F., Alegre, J., Ceresuela, J.L., 2005. The rhizobia nodulating shrubs for revegetation of arid lands: Isolation of native strains and specificity of the plant-rhizobia interaction by cross inoculation tests. *Arid Land Research and Management* 19, 307–326.
- Howieson, J.G., Loi, A., Carr, S.J., 1995. *Biserrula pelecinus* L. – a legume pasture species with potential for acid, duplex soils which is nodulated by unique root-nodule bacteria. *Australian Journal of Agricultural Research* 46, 997–1009.
- Hung, P.Q., Kumar, S.M., Govindsamy, V., Annapurna, K., 2007. Isolation and characterization of endophytic bacteria from wild and cultivated soybean varieties. *Biology and Fertility of Soils* 44, 155–162.
- Ibáñez, F., Angelini, J., Taurian, T., Tonelli, M.L., Fabra, A., 2009. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. *Systematic and Applied Microbiology* 32, 49–55.
- Imran, A., Hafeez, F.Y., Frühling, A., Schumann, P., Malik, K.A., Stackebrandt, E., 2010. *Ochrobactrum ciceri* sp. nov., isolated from nodules of *Cicer arietinum*. *International Journal of Systematic and Evolutionary Microbiology* 60, 1548–1553.
- Kuklinsky-Sobral, H.L., Araujo, W.L., Mendes, R., Pizzirani-Kleiner, A.A., Azevedo, J.L., 2005. Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant and Soil* 273, 91–99.
- Kuklinsky-Sobral, J., Araujo, W.L., Mendes, R., Gerdali, I.O., Pizzirani-Kleiner, A.A., Azevedo, J.L., 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology* 6, 1244–1251.
- Lambinon, J., Langhe, J.D., Delvesalle, L., Duvinneaud, J., 1998. Flora van België, het Groothertogdom Luxemburg, Noord-Frankrijk en de aangrenzende gebieden, Derde druk ed. De Nationale Plantentuin van België, Meise.
- Laranjo, M., Alexandre, A., Rivas, R., Velazquez, E., Young, J.P.W., Oliveira, S., 2008. Chickpea rhizobia symbiosis genes are highly conserved across multiple *Mesorhizobium* species. *FEMS Microbiology Ecology* 66, 391–400.
- Laranjo, M., Machado, J., Young, J.P.W., Oliveira, S., 2004. High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. *FEMS Microbiology Ecology* 48, 101–107.
- Li, Q.Q., Wang, E.T., Chang, Y.L., Zhang, Y.Z., Zhang, Y.M., Sui, X.H., Chen, W.F., Chen, W.X., 2010. *Ensifer (Sinorhizobium) sojae* sp. nov., isolated from root nodules of *Glycine max* grown in saline-alkaline soils in Hebei province of China. *International Journal of Systematic and Evolutionary Microbiology* 61, 1981–1988.
- Lin, D.X., Wang, E.T., Tang, H., Han, T.X., He, Y.R., Guan, S.H., Chen, W.X., 2008. *Shinella kummerowiae* sp. nov., a symbiotic bacterium isolated from root nodules of the herbal legume *Kummerowia stipulacea*. *International Journal of Systematic and Evolutionary Microbiology* 58, 1409–1413.
- Mantelin, S., Fischer-Le Saux, M., Zakhia, F., Bena, G., Bonneau, S., Jeder, H., de Lajudie, P., Cleyet-Marel, J.C., 2006. Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiense* sp. nov., *Phyllobacterium leguminum* sp. nov. and *Phyllobacterium brassicacearum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 56, 827–839.
- Moulin, L., Bena, G., Boivin-Masson, C., Stepkowski, T., 2004. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. *Molecular Phylogenetics and Evolution* 30, 720–732.
- Moulin, L., Munive, A., Dreyfus, B., Boivin-Masson, C., 2001. Nodulation of legumes by members of the beta-subclass of Proteobacteria. *Nature* 411, 948–950.
- Muresu, R., Maddau, G., Delogu, G., Cappuccinelli, P., Squartini, A., 2010. Bacteria colonizing root nodules of wild legumes exhibit virulence-associated properties of mammalian pathogens. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 97, 143–153.
- Muresu, R., Polone, E., Sulas, L., Baldan, B., Tondello, A., Delogu, G., Cappuccinelli, P., Alberghini, S., Benhizia, Y., Benhizia, H., Benguedouar, A., Mori, B., Calamassi, R., Dazzo, F.B., Squartini, A., 2008. Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes. *FEMS Microbiology Ecology* 63, 383–400.
- Nangul, A., Moot, D.J., Brown, D., Ridgway, H.J., 2013. Nodule occupancy by *Rhizobium leguminosarum* strain WSM1325 following inoculation of four annual *Trifolium* species in Canterbury, New Zealand. *New Zealand Journal of Agricultural Research* 56, 215–223.
- Ogutcu, H., Algur, O.F., Elkoca, E., Kantar, F., 2008. The determination of symbiotic effectiveness of *Rhizobium* strains isolated from wild chickpeas collected from high altitudes in erzurum. *Turkish Journal of Agriculture and Forestry* 32, 241–248.
- Palaniappan, P., Chauhan, P.S., Saravanan, V.S., Anandham, R., Sa, T.M., 2010. Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lepedeza* sp. *Biology and Fertility of Soils* 46, 807–816.
- Parmar, N., Dadarwal, K.R., 1999. Stimulation of nitrogen fixation and induction of flavonoid-like compounds by rhizobacteria. *Journal of Applied Microbiology* 86, 36–44.
- Pearson, W.R., 1990. Rapid and sensitive sequence comparison with Fastp and Fasta. *Methods in Enzymology* 183, 63–98.

- Perret, X., Staehelin, C., Broughton, W.J., 2000. Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews* 64, 180–201.
- Pule-Meulenberg, F., Belane, A.K., Krasova-Wade, T., Dakora, F.D., 2010. Symbiotic functioning and bradyrhizobial biodiversity of cowpea (*Vigna unguiculata* L. Walp.) in Africa. *BMC Microbiology* 10, 89.
- Putnam, M.L., Miller, M.L., 2007. *Rhodococcus fascians* in herbaceous perennials. *Plant Disease* 91, 1064–1076.
- R Core Team, 2013. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Radl, V., Simoes-Araujo, J.L., Leite, J., Passos, S.R., Martins, L.M.V., Xavier, G.R., Rumjanek, N.G., Baldani, J.I., Zilli, J.E., 2014. *Microvirga vignae* sp. nov., a root nodule symbiotic bacterium isolated from cowpea grown in semi-arid Brazil. *International Journal of Systematic and Evolutionary Microbiology* 64, 725–730.
- Rajendran, G., Sing, F., Desai, A.J., Archana, G., 2008. Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. *Bioresource Technology* 99, 4544–4550.
- Rivas, R., Willems, A., Subba-Rao, N.S., Mateos, P.F., Dazzo, F.B., Kroppenstedt, R.M., Martínez-Molina, E., Gillis, M., Velázquez, E., 2003. Description of *Devosia neptuniae* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. *Systematic and Applied Microbiology* 26, 47–53.
- Robledo, E.A., Kmiecik, K., Oplinger, E.S., Nienhuis, J., Triplett, E.W., 1998. Trifoliotoxin production increases nodulation competitiveness of *Rhizobium etli* CE3 under agricultural conditions. *Applied and Environmental Microbiology* 64, 2630–2633.
- Sawada, H., Kuykendall, L.D., Young, J.M., 2003. Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. *Journal of General and Applied Microbiology* 49, 155–179.
- Shiraishi, A., Matsushita, N., Hougetsu, T., 2010. Nodulation in black locust by the Gammaproteobacteria *Pseudomonas* sp. and the Betaproteobacteria *Burkholderia* sp. *Systematic and Applied Microbiology* 33, 269–274.
- Sturz, A.V., Christie, B.R., Matheson, B.G., Nowak, J., 1997. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils* 25, 13–19.
- Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., Dreyfus, B., 2001. Methylophilic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology* 183, 214–220.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum Likelihood, evolutionary distance, and maximum Parsimony methods. *Molecular Biology and Evolution* 28, 2731–2739.
- Tariq, M., Hameed, S., Yasmeen, T., Zahid, M., Zafar, M., 2014. Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (*Pisum sativum* L.). *World Journal of Microbiology and Biotechnology* 30, 719–725.
- Trujillo, M.E., Willems, A., Abril, A., Planchuelo, A.M., Rivas, R., Ludena, D., Mateos, P.F., Martínez-Molina, E., Velázquez, E., 2005. Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Applied and Environmental Microbiology* 71, 1318–1327.
- Valverde, A., Velázquez, E., Fernandez-Santos, F., Vizcaino, N., Rivas, R., Mateos, P.F., Martínez-Molina, E., Igual, J.M., Willems, A., 2005. *Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. *International Journal of Systematic and Evolutionary Microbiology* 55, 1985–1989.
- Valverde, A., Velázquez, E., Gutierrez, C., Cervantes, E., Ventosa, A., Igual, J.M., 2003. *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. *International Journal of Systematic and Evolutionary Microbiology* 53, 1979–1983.
- Van Landuyt, W., Hoste, I., Vanhecke, L., Vercruyse, W., Van Den Bremt, P., De Beer, D., 2006. Atlas van de flora van Vlaanderen en het Brussels Gewest. INBO and Nationale Plantentuin van België, Brussels.
- Van Landuyt, W., Vanhecke, L., Hoste, I., Bauwens, D., 2011. Do the distribution patterns of vascular plant species correspond to biogeographical classifications based on environmental data? A case study from northern Belgium. *Landscape and Urban Planning* 99, 93–103.
- Vancanneyt, M., Mengaud, J., Cleenwerck, I., Vanhoneracker, K., Hoste, B., Dawyndt, P., Degivry, M.C., Ringuelet, D., Janssens, D., Swings, J., 2004. Reclassification of *Lactobacillus kefirgranum* Takizawa et al. 1994 as *Lactobacillus kefirnofaciens* subsp. *kefirgranum* subsp. nov. and emended description of *L. kefirnofaciens* Fujisawa et al. 1988. *International Journal of Systematic and Evolutionary Microbiology* 54, 551–556.
- Versalovic, J., Schneider, M., De Bruijn, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in molecular and cellular biology* 5, 25–40.
- Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil* 255, 571–586.
- Vidal, C., Chantreuil, C., Berge, O., Maure, L., Escarpe, J., Bena, G., Brunel, B., Cleyet-Marel, J.C., 2009. *Mesorhizobium metallidurans* sp. nov., a metal-resistant symbiont of *Anthyllus vulneraria* growing on metalcolous soil in Languedoc, France. *International Journal of Systematic and Evolutionary Microbiology* 59, 850–855.
- Vincent, J.M., 1970. A Manual for the Practical Study of the Root-nodule Bacteria, International Biological Programme Handbook, 15 ed. Blackwell Scientific Publications, Oxford, UK.
- Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73, 5261–5267.
- Ward Jr., J.H., 1963. Hierarchical grouping to optimize an objective function. *Journal of the American statistical association* 58, 236–244.
- Wilson, R.A., Handley, B.A., Beringer, J.E., 1998. Bacteriocin production and resistance in a field population of *Rhizobium leguminosarum* biovar *Viciae*. *Soil Biology & Biochemistry* 30, 413–417.
- Zakhia, F., Jeder, H., Willems, A., Gillis, M., Dreyfus, B., de Lajudie, P., 2006. Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for *nifH*-like gene within the genera *Microbacterium* and *Starkeya*. *Microbial Ecology* 51, 375–393.
- Zhang, F., Dashti, N., Hynes, R.K., Smith, D.L., 1996. Plant growth promoting rhizobacteria and soybean [*Glycine max* (L) Merr] nodulation and nitrogen fixation at suboptimal root zone temperatures. *Annals of Botany* 77, 453–459.
- Zurdo-Pineiro, J.L., Rivas, R., Trujillo, M.E., Vizcaino, N., Carrasco, J.A., Chamber, M., Palomares, A., Mateos, P.F., Martínez-Molina, E., Velázquez, E., 2007. *Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain. *International Journal of Systematic and Evolutionary Microbiology* 57, 784–788.